VARIATION OF CAROTENE CONTENT OF ALFALFA

by

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INTRODUCTION

Although vitamin A is a substance of utmost importance in animal nutrition, no direct evidence has been presented as yet showing that any one of its four common precursors: alpha-carotene, beta-carotene, gamma-carotene, and cryptoxanthin, can be synthesized during animal metabolism. Vitamin A is not known to occur in the plant kingdom. Beta-carotene generally is more plentiful in plant tissue than the other potential sources of vitamin A. This is fortunate, for its molecular structure gives it twice the vitamin A potency as is possessed by the other plant pigments which can serve as precursors to vitamin A.

Alfalfa meal is in large demand as a source of vitamin A in manufactured animal feeds. This is illustrated by the fact that 60 of the 72 alfalfa dehydration plants operating in Kansas in 1948 have been established since 1944. Considerable experimental work has been done to determine the effects of various environmental factors upon the increase and preservation of carotene in alfalfa. It has been shown also that the amount of carotene in alfalfa varies with the genetic make-up of the plant, but the actual manner of inheritance has not been demonstrated.

The object of this study was to observe the variations in carotene content in alfalfa under conditions of controlled breeding, and to present preliminary information concerning the manner in which carotene content in alfalfa is inherited.

REVIEW OF LITERATURE

Titus (1942) reported that histological examination of chicken tissue has indicated that vitamin A functions in the nourishment and repair of both internal and external epithelial tissue of the body. Eichhorn, et al. (1942) have shown that when vitamin A is not present to function in rejuvenation, normal epithelial tissue soon becomes keratinized (horny) and as a result, bacterial infection, xerophthalmia, and other symptoms appear, which, if not corrected by the addition of vitamin A to the ration, will cause ultimate disablement or death of the animal concerned. White scours of calves, anasarca and edema of cattle, and the loss of reproductive ability are all indicators of a greater or lesser degree of vitamin A deficiency.

Hackerott (1946) has shown that moisture conditions under which alfalfa has been grown has a marked effect upon its carotene content. Plants grown in the greenhouse and in the field with the soil moisture content approaching drought conditions were significantly higher in carotene than those grown in soil near field moisture holding capacity. He, along with other workers, has also shown that fertilizers in general do not increase carotene content of alfalfa unless soil fertility is very low. Lime and nitrogen applications under greenhouse conditions caused an increase in total carotene, but this evidently was due to an increase in the leaf-stem ratio. According to Ham and Tysdal (1946), the leaves contain 77.1 percent of the total carotene

of the alfalfa plant. This statement agrees in general with the findings of Hauge (1934) who reported that the leaves contained 10 to 14 times as much carotene as the stems.

Any factor which causes a chlorosis of the plant leaf will reduce its carotene content. Johnson (1938) found that alfalfa which had been protected from leaf hopper attack by dusting had significantly higher carotene content at harvest and after several months' storage in a warm attic than alfalfa not dusted and severely yellowed by leaf hopper attack. This evidence was supported by Ham and Tysdal (1946) who noted that badly-yellowed strains of alfalfa contained less carotene than strains which had some resistance to leaf hopper damage. Hamner (1945), working with turnip greens, decided that any nutrient deficiency which would cause a chlorotic condition of the leaves would have a detrimental effect upon the carotene content of the plant.

With respect to stage of growth, Hauge (1934) has demonstrated that young alfalfa 10 to 12 inches high has greater vitamin A potency than alfalfa in the bloom stage. This agrees with the results of Ham and Tysdal (1946) who found that the new, more succulent alfalfa growth was higher in carotene than the older growth. They reported that the stems of the younger material did not vary greatly in carotene content from the stems of older plants, but the leaves of the former were considerably higher in carotene than those of the latter. Meenan (1945), however, reported that carotene is continually built up in the leaves of the alfalfa plant until the blooming period. He found more carotene in the

leaves of older plants than he did in young material and considered that the loss in total carotenoid content of the plant as it matures was probably due to the loss of leaves.

Evidence has been presented that the carotene content of several plants is under genetic control. Le Rosen, et al. (1941), working with the factors R and r, governing respectively the red and yellow flesh color of tomatoes, found that dominant R was accompanied by high lycopene content of red tomatoes as well as by increases of yellow carotenoid pigments. They concluded that this gene might have a pleiotropic effect or that a series of genes controlling carotenoid content might be closely linked to flesh color of tomatoes. Kohler, et al. (1947) obtained yellow high beta-carotene tomatoes by backcrossing selections from the cross Baltimore x F1 (Rutgers x Lycopersicum hirsutum--P. I. 126445) to commercial varieties and decided that there were only a few major factors involved in the control of beta-carotene. It appeared to these authors that beta-carotene was produced at the expense of lycopene since these deep-orange, high beta-carotene segregates did not have a higher total carotenoid content than the red, low beta-carotene parent.

The relationship between the genetic make-up of corn and its vitamin A and carotene content has been the objective of several investigators. The earliest work reported is that of Steenbock and Boutwell (1920) who found that the deep yellow seeds on ears segregating for white and yellow endosperm were higher in vitamin

A than a mixture of the white and pale yellow seeds. This condition was substantiated by Hauge and Trost (1928) and by Hauge (1930) who reported that they could find this fat-soluble substance only in those corn kernels with yellow endosperm, even when both white and yellow kernels were produced on the same ear. In an article published in 1930, these same authors came to the conclusion that the vitamin A activity of yellow dent corn was controlled by the same factors which gave yellow endosperm. Mangelsdorf and Fraps (1931) supported this conclusion when they obtained evidence that the amount of vitamin A in the corn kernel was directly related to the number of Y genes present for yellow endosperm expression. They suggested a chemical reaction which might have taken place between these genes and some substance in the endosperm of white and yellow corn. They found white corn to be very low in vitamin A activity, but as factors for yellow endosperm were introduced, each gene induced the formation of approximately 2.5 units of vitamin A per gram of seed, indicating the fact that the carotenoid pigments might be intermediate products of the reaction. Randolf and Hand (1938, 1940) obtained similar results. They found that upon doubling the chromosome number of diploid corn, there was a 40 percent increase in total carotenoid content accompanied by a proportionate increase in vitamin A activity. This, along with an increase in cell volume of 3.6 times over that of the diploid plants, resulted in a five-fold increase in the amount of carotene per cell. An accumulation

effect was noted following chromosome doubling, with each gene present for yellow endosperm expression being responsible for the formation of 2.5 times more carotene than in the diploid cells, even though the number of genes per unit volume was greater in the diploid than in the tetraploid cells. Johnson and Miller (1939) reported similar xenia effects and also came to the conclusion that the carotene content of the foliage of the corn plant was independent of the factors responsible for that of the corn kernel, since no significant correlation between the two was noted.

Studies carried on with carrots by Emsweller, et al. (1935) have shown that in inbred lines carotene content becomes more uniform. These authors considered that they had obtained segregates which would be useful in breeding high, uniform carotene content into commercial varieties.

Several workers have shown definitely that carotene content in alfalfa is controlled by heritable factors. Ham and Tysdal (1946) obtained strains and hybrids which they considered to differ inherently in carotene content, since the variations shown by the progeny were consistent when factors detrimental to carotene content were held under control. They found high leaf percentage to be an indicator of high carotenoid content in general. They thought also that high carotene might be associated with dark green leaf color. Hackerott (1946) found differences in total carotenoid content between strains and clones of alfalfa and obtained a significant correlation between the carotene content of polycross progeny and the carotene content of their maternal par-

ents. He decided that these differences could not be attributed completely to difference in percentages of leaves between plants, but was at least partially due to variation in concentration of carotene in the plant tissue. He concluded that carotene content in alfalfa is evidently inherited and that the polycross method as used in alfalfa breeding is a satisfactory one by which the ability of clones to transmit their genetic factors for high carotene content might be determined.

MATERIALS AND METHODS

All alfalfa plants used in this work had, as their origin, the original stock of Kansas Common alfalfa seed from which the selection was made which resulted in Buffalo alfalfa. This study was begun with 9 groups of plants of the following parentage: (1) the selfed progeny of K30-3, K30-5, K30-21 (C-91), K30-55 (C-95), K30-58 (C-96), K30-63 (C-97), K30-64 (C-98), (2) the F1 populations of the cross K30-21 x K30-55 and its reciprocal.[1] These plants were selected for study on the basis of observations made by Hackerott (1946). The crosses were made by Albert Davis

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[1] K stands for Kansas selection. The first digit (3) represents the year 1943 in which the selection was made, while the second digit (0) indicates this selection was material newly introduced into the breeding program and of unknown potentialities. The last number represents a certain selection as listed in the permanent record book. The "C" number is the official designation assigned by the Div. of Forage Crops and Diseases, B.P.I., S. and A.E. Method of designating alfalfa plants follows the suggestion of Newell, L. C. and Tysdal, H. M. Numbering and note-taking systems for use in the improvements of forage crops, Amer. Soc. Agron. Jour. 37:736-749. 1945.

in the greenhouse in 1948.[2] With the exception of K30-3 and K30-5, these plants are all a part of the alfalfa breeding program carried on at the Kansas Agricultural Experiment Station in cooperation with the Division of Forage Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering, U. S. D. A. Future reference will be made to the parental plants used in this experiment using only the selection number.

The progenies of these plants were started in flats in the greenhouse and transferred to the field in April, 1948. Planting was done at random in 36-inch rows with plants 18 inches apart in a uniformly sandy loam soil. An overhead sprinkling system was installed in order to maintain an optimum and uniform moisture content of the soil throughout the season. The plot was fertilized with 10-20-0 applied at the rate of 300 pounds per acre, and sprayed at regular intervals with a suspension of 50 percent wettable DDT in water in order to keep the population of leaf hopper (Empoasca fabae Harr) and other foliage-damaging insects at a minimum. Three cuttings were taken during the growing season. At the time of the second and third cuttings, notes were taken on leaf color, blossom color, disease, leaf retention, stage of maturity and leafiness.

Carotene analysis was made by the method of Mitchell and King (1948). Immediately after cutting, the samples were transferred to the laboratory where they were blanched in an autoclave

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[2] A former graduate student of Kansas State College now located at the University of Arkansas, Fayetteville, Arkansas.

for 10 minutes at 10 pounds pressure in order to inactivate the enzyme system responsible for the destruction of carotene in wilted alfalfa. After blanching, the samples were dried in a circulating hot air oven at 65° F. for a period of four to five hours. The plant material was ground in a Wiley mill to pass through a 20-mesh screen, thoroughly mixed and sampled. These samples were stored in paper envelopes at -20° C. to prevent carotene loss due to atmospheric oxidation until carotene analysis was carried out. This analysis consisted of the extraction of a one gram sample of the dried plant material overnight in a solution of one part acetone to two parts Skellysolve B. The carotenes were separated from the other plant pigments using an absorption column consisting of two parts Hyflo-Supercel and one part magnesium oxide, and were eluted from the column by the addition of a four to five percent solution of acetone in Skellysolve B. The various carotene fractions were not separated. Color density measurements were obtained by use of a Beckman quartz spectrophotometer. These were converted and are reported as milligrams of carotene per 100 grams of dry matter.

EXPERIMENTAL RESULTS

Statistical tests were made to determine the variation present both between plant groups and among the plants of any single group. Only one carotene sample was collected from each plant per cutting; the variation within a sampled line could not be determined with accuracy as the different sampling dates could not

be said to constitute replications.

An important source of variation was that attributable to differences in the stage of maturity of the plants at the time of harvest, particularly in the third cutting. Figures 1 and 2 represent the regression of carotene content on stage of maturity for the entire population of plants. Regression coefficients of -1.1000, .1000, and -1.3290 were calculated for the first, second, and third cuttings, respectively. That for the first cutting approached significance at the 5-percent level while -1.3290 found for the third cutting was significant at the 1-percent level.

Some plants were lost after the first and second cuttings as a result of lack of vigor; thus, carotene values for all plants of each group were not obtained for all cuttings. The selfed progenies of plants 1 and 63 were so depleted that no attempt was made to make individual analyses of these groups. Only three individuals were available from plant 64 at the beginning of the experiment; therefore, this group was also omitted from these calculations.

As only one reading per cutting was available for each plant, analysis of variance on the basis of total carotene produced during the season was calculated, eliminating those plants from consideration for which three readings were not obtained. Table 1 presents the results obtained from these calculations. The most outstanding source of variation in carotene content of each plant group was that attributable to differences between cuttings. In each case, a highly significant calculated F value was obtained.

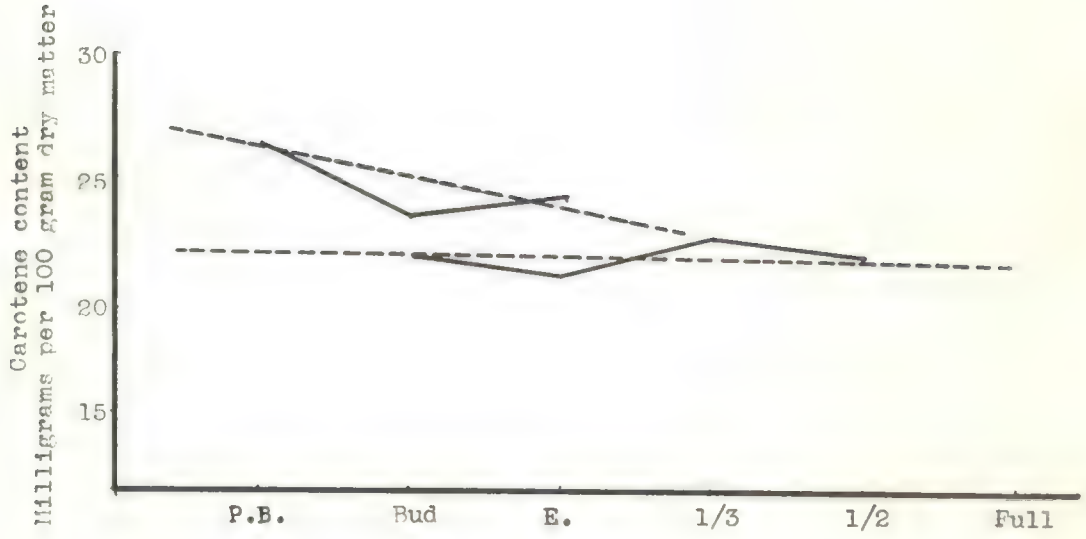

Fig. 1. Mean carotene content of alfalfa plants in various stages of maturity for entire population for first and second cutting.

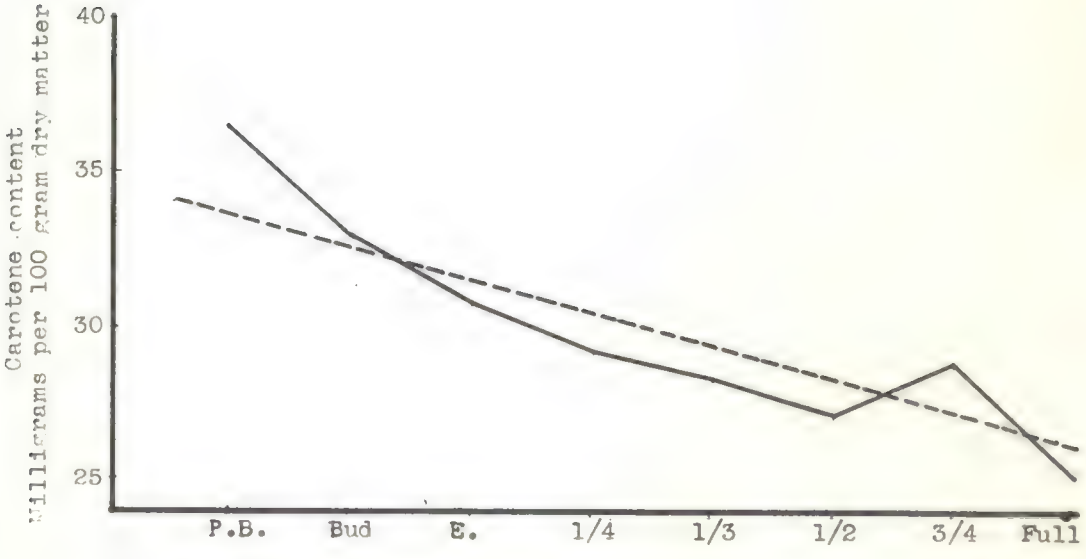

Fig. 2. Mean carotene content of alfalfa plants in various stages of maturity for entire population for third cutting.

Table 1. Analysis of variance of carotene content of four inbred lines and two reciprocal crosses as individual groups.

Group	Factor	D. F.	S.S.	V	Cal.F.	Required for significance .05	.01
21 selfed	Total	80	2,459.86				
	Between plants	26	815.01	31.35	4.403**	1.69	2.10
	Between cuttings	2	1,274.42	637.21	89.495	3.18	5.06
	Error	52	370.43	7.12			
55 selfed	Total	26	796.01				
	Between plants	8	220.96	27.62	1.8738	2.59	3.89
	Between cuttings	2	339.21	169.61	11.5067	3.63	6.23
	Error	16	235.84	14.74			
21x55 (F1)	Total	26	281.15				
	Between plants	8	130.06	16.26	4.7823**	2.59	3.89
	Between cuttings	2	96.68	48.34	14.2175	3.63	6.23
	Error	16	54.41	3.40			
55x21 (F1)	Total	56	885.15				
	Between plants	18	259.66	14.43	1.83	1.90	2.48
	Between cuttings	2	342.27	171.14	21.76	3.27	5.25
	Error	36	283.22	7.87			
5 selfed	Total	14	323.51				
	Between plants	4	18.81	4.70	1.103	3.84	7.01
	Between cuttings	2	270.60	135.30	31.76		
	Error	8	34.10	4.26			
58 selfed	Total	83	1,905.62				
	Between plants	27	493.37	18.27	1.4196	1.63	2.18
	Between cuttings	2	717.32	358.66	27.8679	3.15	5.06
	Error	54	694.93	12.87			

**Highly significant

This agrees with the results of Hackerott (1946) who found that carotene content of alfalfa is generally higher in the spring and early summer, and in the fall, than it is in midsummer. The means of the plant groups used in this experiment compiled on the basis of one cutting each on June 6, July 12, and August 26, indicate that, on the average, the greatest amount of carotene was found in the plant material collected in the August cutting, and the least in the July cutting (Fig. 3). Moisture content of the soil was kept as uniform as possible during the course of these studies; therefore, it is doubtful that these differences in carotene content can be attributed to a varying moisture supply.

Data presented in Table 1 show that there was no significant difference among the plants of the selfed progenies of plants 5, 55, and 58, and the hybrid progeny of the cross 55 x 21. An F value of 1.83 was calculated for the latter group with F = 1.90 required for significance at the 5-percent level. Analysis of the data collected for the selfed progeny of plant 21 and the F1 generation of the cross 21 x 55 indicated that there were highly significant differences among the plants in each of these two populations.

Table 2 shows that the selfed progeny of plant 21 contained individuals with the highest carotene content. Plant 147 and plant 53 had 101.5 and 100.5 milligrams of total carotene respectively and were the highest individuals produced in any population under study. Three groups, the hybrids of the two crosses 21 x 55 and 55 x 21, and the selfed progeny of plant 55 produced no indi-

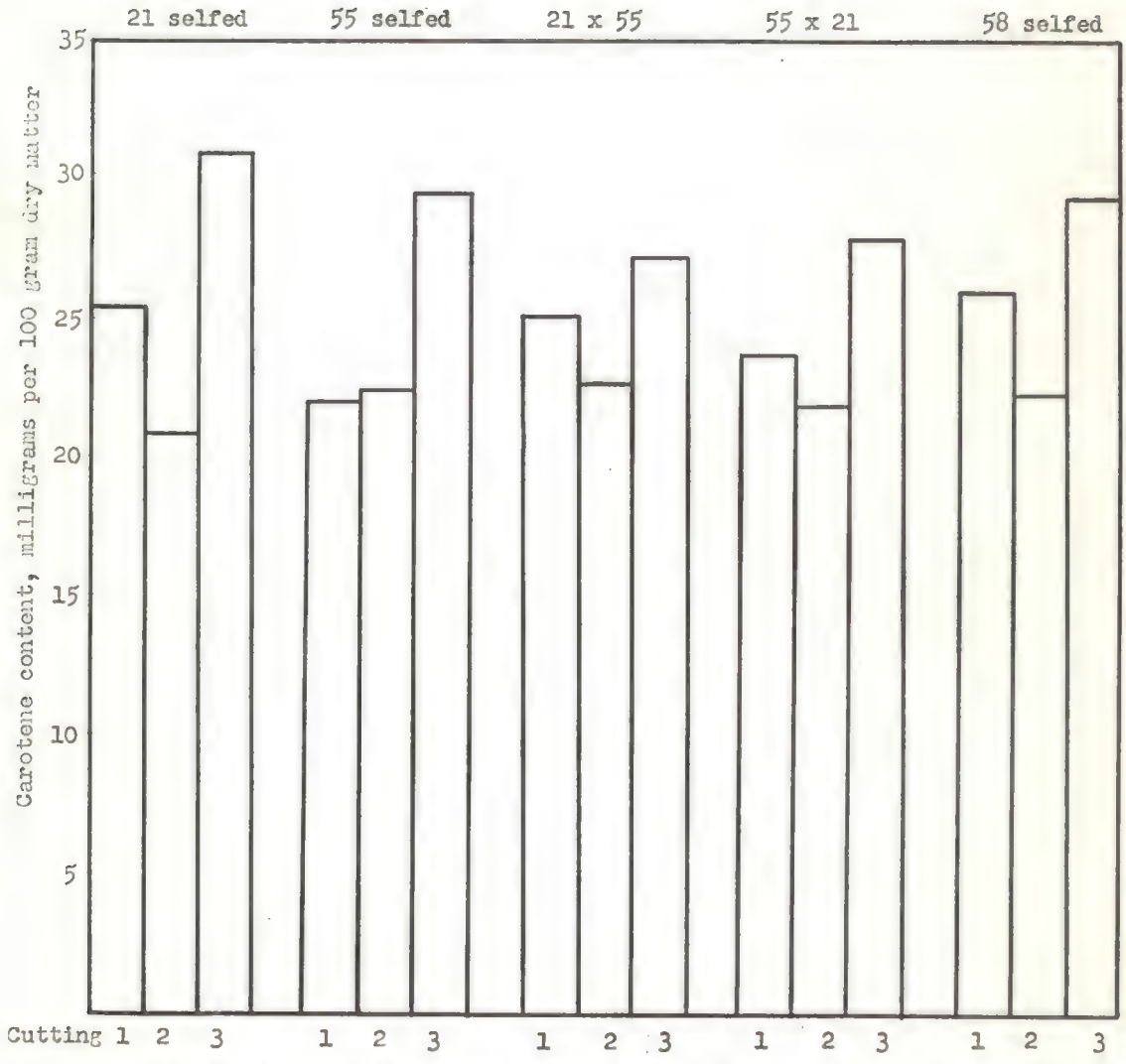

Fig. 3. Mean carotene content of the progeny of three inbred lines and two hybrid populations for each cutting date.

Table 5. Carotene content and rank of individual plants of four inbred lines and two hybrid progenies.

Plant No.	1st cutting		2nd cutting		3rd cutting		Total	
	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank
21 selfed								
147	35.3	1	25.0	2	39.9	2	101.5	1
148	29.6	2	21.6	8	35.2	6	86.4	5
25	29.6	2	23.1	6	37.9	3	90.6	3
59	27.6	4	20.0	13	30.6	11	78.2	11
42	27.3	5	18.8	20	21.3	26	67.8	24
145	27.2	6	19.6	16	26.3	23	73.1	18
14	26.9	7	25.3	3	34.5	7	86.7	4
53	26.9	7	30.9	1	42.7	1	100.5	2
21	26.6	9	24.2	4	29.6	13	80.4	8
26	26.4	10	23.0	7	28.2	14	78.3	10
28	26.0	11	19.5	17	27.0	22	72.5	15
87	26.0	11	21.6	8	30.0	12	77.6	12
84	25.4	13	20.0	13	36.5	5	81.9	7
86	25.4	13	17.6	22	28.9	15	71.9	16
62	25.1	15	19.2	15	30.9	10	75.2	13
49	25.0	16	21.6	8	32.9	9	79.5	9
5	24.7	17	15.2	26	31.0	9	70.9	19
43	24.6	18	16.9	24	25.2	25	66.7	23
117	24.5	19	21.5	12	25.6	24	71.6	17
52	24.3	20	23.5	5	36.7	4	84.2	6
44	23.6	21	19.3	19	27.2	19	70.1	20
120	22.6	22	15.8	27	27.4	18	65.8	26
4	22.2	23	17.5	23	27.1	21	66.8	22
125	20.4	24	19.4	18	28.5	17	68.3	21
104	20.3	25	18.7	21	27.2	19	66.2	25
57	19.6	26	21.6	8	29.6	13	70.8	19
Mean	25.1		20.8		30.7		77.08	

Table 2. (cont.).

Plant No.	1st cutting		2nd cutting		3rd cutting		Total	
	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank
				55 x 21				
41	28.8	1	23.0	6	29.1	7	80.9	2
32	27.3	2	21.0	12	24.9	17	73.2	10
7	25.7	3	21.6	10	30.1	6	77.4	8
133	25.4	4	22.8	7	26.1	10	74.3	9
92	24.8	5	19.3	17	25.0	16	69.1	14
138	24.7	6	22.7	8	25.4	14	72.8	11
100	24.2	7	28.1	1	26.1	10	78.4	6
34	24.0	8	17.4	18	25.2	15	66.6	17
31	23.8	9	27.1	2	33.6	2	84.5	1
70	23.4	10	25.0	5	31.0	5	79.4	5
38	23.3	11	21.4	11	35.0	1	79.7	4
91	23.1	11	25.5	4	31.4	4	80.2	3
48	22.7	13	22.5	9	26.0	13	71.2	13
149	22.4	14	13.8	19	24.2	18	60.4	19
10	21.8	15	20.9	13	28.7	8	71.4	12
123	21.7	16	20.1	15	21.6	19	63.4	18
85	21.0	17	20.3	14	26.1	10	67.4	16
114	19.7	18	20.0	16	27.3	9	67.5	15
52	19.5	19	26.0	3	31.8	3	76.1	7
Mean	23.6		22.1		27.8		73.47	
				55 Salford				
29	27.7	1	17.7	9	30.8	1	84.3	2
16	24.5	2	24.7	3	31.6	4	80.8	3
131	23.8	3	23.1	4	30.2	5	77.6	5
24	23.1	4	20.3	7	33.2	3	76.6	6

Table I. (cont.).

Plant No.	1st cutting		2nd cutting		3rd cutting		Total	
	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank
56	22.7	5	26.6	1	37.2	2	86.5	1
105	22.1	6	20.7	6	29.0	6	71.8	7
142	21.7	7	22.4	5	20.5	8	64.6	8
128	20.1	8	20.8	8	19.2	9	59.6	9
19	20.2	8	26.0	2	27.9	7	74.1	6
Mean	22.9		24.1		29.8		75.99	
				21 x 51				
63	25.8	1	24.2	3	30.3	2	81.0	2
66	25.2	2	26.6	1	32.1	1	83.5	1
12	24.7	3	22.7	5	27.4	4	74.8	4
137	24.7	3	25.9	2	24.2	9	74.8	4
126	24.1	5	20.9	8	24.3	8	69.3	8
90	23.7	6	22.9	4	26.3	5	72.9	6
110	23.4	7	21.6	6	27.4	4	72.4	6
150	23.3	8	21.5	7	25.9	6	70.7	7
39	21.0	9	19.3	9	24.6	7	60.9	9
Mean	24.0		22.7		27.2		73.81	
				18 Selfed				
83	31.3	1	24.3	6	32.7	9	88.3	2
141	30.2	2	27.4	3	25.3	23	83.3	4
82	30.6	3	22.7	11	26.8	16	82.1	6
18	28.5	4	17.5	27	37.6	2	81.6	7
2	28.4	5	18.2	25	27.6	20	74.2	19
99	28.3	6	32.7	1	38.1	1	99.1	1
108	28.1	7	18.3	24	29.8	12	76.7	16

Table 2. (concl.).

Plant No.	1st cutting		2nd cutting		3rd cutting		Total	
	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank	Carotene content	Rank
8	28.1	7	21.0	16	31.0	10	80.1	12
111	27.5	9	21.3	14	34.1	4	82.9	5
70	27.3	10	23.2	10	24.8	24	76.0	17
33	27.0	11	19.8	22	33.7	5	80.5	10
7	26.6	12	21.0	16	24.7	25	72.3	21
64	26.3	13	22.1	12	33.3	6	81.7	8
73	25.8	14	20.0	21	27.9	18	73.7	20
78	25.6	15	20.1	20	36.2	3	81.9	7
151	25.6	15	21.3	15	23.4	26	70.3	24
119	25.1	17	20.9	19	25.6	22	71.6	23
58	25.0	18	28.5	2	27.1	21	80.6	9
74	24.9	19	17.3	28	27.9	18	70.1	25
68	24.5	20	26.2	4	28.0	16	78.7	13
17	23.9	21	17.4	27	29.4	14	78.1	15
77	23.6	22	24.3	7	30.6	11	78.5	14
140	23.0	23	21.0	16	20.7	27	64.7	27
143	23.0	23	19.4	23	20.6	28	63.0	28
9	22.7	25	24.6	6	33.2	7	80.5	10
20	21.1	26	24.3	8	29.0	15	74.4	18
11	21.0	27	18.4	26	31.4	8	72.2	22
197	17.2	28	21.6	13	29.4	13	68.0	26
Mean	25.7		22.3		29.4		77.43	
				I Selfed				
13	23.0	1	18.9	2	30.6	2	72.5	1
112	21.2	2	17.1	4	34.1	1	63.1	4
81	18.7	3	16.7	5	27.5	4	63.9	3
69	18.8	4	20.0	1	27.6	3	66.4	2
76	18.7	5	18.5	3	30.9	1	68.1	2
Mean	20.4		18.2		24.1		66.8	

viduals which were exceptionally high in total carotene content, the best being 85.5, 84.5, and 83.5 milligrams of total carotene, respectively, for the three groups. Plant 37 was the best from the selfed progeny of plant 33, showing a total of 77.1 milligrams of carotene produced in three cuttings; while the plant with the highest carotene from the selfed progeny of plant 5 was plant 13 with 72.6 milligrams of carotene.

The mean total carotene produced per plant in each group is presented in Table 2. The selfed progeny from plant 33 showed the highest mean (77.43 milligrams), followed closely by the 21 group with a mean of 77.08 milligrams of carotene. The selfed progeny of plant 37 was third highest with a mean total of 77.09 milligrams. The mean total carotene per plant produced by the hybrid progenies of the two crosses was 73.47 and 73.81 milligrams for 37 x 21 and 21 x 33, respectively. The progenies from plant 5 were the lowest of the groups studied, producing an average total carotene content of 66.8 milligrams.

Table 3 shows the total carotene produced by the selfed progeny of plant 21 and the hybrids from the cross 21 x 33, with individual plants arranged in the order of their rank. A difference of 4.60 mgs of carotene at the 5-percent level and 6.32 mgs at the 1-percent level is required for significance in the inbred population. For the group of F1's, the difference between plants required for significance is 3.07 mgs at the 5-percent level and 4.22 mgs of carotene at the 1-percent level. The data indicate that the plants of these two strains differ significantly from

Table 3. Total carotene produced by selfed progeny of plant 21 and the F1's of the cross 21 x 55 with differences between the plants calculated.

21 Selfed			21 x 55		
Plant No.	Mg. carotene	Difference*	Plant No.	Total carotene	Difference**
147	101.5		66	85.5	
53	100.5	1.0	63	81.0	4.5
25	90.6	9.9	90	74.9	6.1
14	86.7	3.9	12	74.8	.1
148	86.4	0.3	137	74.8	0
55	84.2	2.2	118	72.4	2.4
84	81.9	2.3	150	70.7	1.7
21	80.4	1.9	126	69.3	1.4
49	79.5	0.9	39	60.9	8.4
26	78.3	1.2			
59	78.2	0.1			
87	77.6	0.6			
62	75.7	1.9			
145	73.1	2.6			
28	72.5	.6			
86	71.9	.6			
117	71.6	.3			
5	70.9	.7			
57	70.8	.1			
44	70.1	.7			
125	68.3	1.8			
42	67.8	.5			
4	66.8	1.0			
45	66.7	.1			
104	66.2	.5			
120	65.8	.4			

*L.S.D. between totals = 4.62 mgs at the 5-percent level and 6.22 mgs at the 1-percent level.

**L.S.D. between totals = 3.07 mgs at the 5-percent level and 4.22 at the 1-percent level.

one another in the total carotene produced in three cuttings.

The correlation between cuttings in regard to constancy of rank in successive cuttings was calculated (Table 4). The selfed progeny of plant 55 and the hybrid populations from the reciprocal crosses 21 x 55 and 55 x 21 showed significant correlation between the first and third, the first and second, and the second and third cuttings, respectively. The first and second cuttings of the cross 21 x 55 showed highly significant correlation while the other two comparisons, first vs. second and second vs. third, closely approached significance at the 5-percent level. The selfed progeny of plant 21 showed significant correlation among all three cuttings, exceeding the 1-percent level of significance when the second and third cuttings were compared. None of the progeny produced by selfing plants 5 or 58 possessed significant correlation from cutting to cutting. Table 2 shows the carotene content of the plants used in the above calculations and their rank in each cutting.

A study of the extreme of variation in individual cuttings of the various groups showed that comparable differences were present which did not show up in the analysis of variance calculations. The fact that these differences were in some cases combined in the totals to form significant variation suggested that at least two sources of variation were present. Genetic segregation in addition to environmental variation would apparently need to be present in order to account for these different degrees of significance.

Table 4. Correlation between cuttings in four inbred lines and two hybrid populations of alfalfa.

Plant grow	Cutting	D.F.	Correlation coefficient	Required for significance[1] .05	.01
55 selfed	1 vs 2	7	-.216	.666	.798
	1 vs 3		.741*		
	2 vs 3		.015		
21 selfed	1 vs 2	24	.455*	.388	.496
	1 vs 3		.483*		
	2 vs 3		.712**		
21x55 (F1)	1 vs 2	7	.891**	.666	.798
	1 vs 3		.567		
	2 vs 3		.625		
55x21 (F1)	1 vs 2	17	.086	.456	.575
	1 vs 3		.059		
	2 vs 3		.512*		
58 selfed	1 vs 2	26	.116	.374	.478
	1 vs 3		.169		
	2 vs 3		.090		
9 selfed	1 vs 2	3	-.208	.878	.959
	1 vs 3		-.142		
	2 vs 3		.467		

[1] Snedecor, G. W., Statistical Methods, 4th Edition, 1946. From Table 7.3, p174).

*Significant

**Highly significant

In order to obtain an indication of the differences in carotene content existing between and among the plant groups used in this experiment, analysis of the group data on the basis of separate cuttings was carried out. Significant differences between groups were found only in the first cutting. Table 5 shows the F values calculated and those necessary for significance at the 1 and 5-percent levels. Allowing for varying sample size (Table 2), differences between means in the first cutting of 1.4-2.6 is necessary for significance at the 5-percent level, while 1.6-2.6 is required at the 1-percent level. Figure 3 shows that wider differences were encountered between the means of the third cutting than of the first; this greater variation was nonsignificant because of the greater error term calculated for the third cutting. According to these data, the variation present among the plants of a given group increased in successive cuttings and the differences observed in the first cutting between the strains used in this experiment disappeared in the second and third cuttings. This increase in variability within groups was studied by comparing the error variance terms calculated for each cutting. Increases in variation in the third cutting over that found in both the second and third cuttings were highly significant. Variation was not significant (P = .08) between the first and second cuttings.

Man and Tysdal (1946) suggested that alfalfa plants with dark green foliage might be higher in carotene content than those of a less intense green color. Notes were taken on intensity of greenness of the plants under observation for the second and third cut-

Table 5. Analysis of variance of carotene content of three inbred lines and two hybrid alfalfa progenies showing the variation caused by dates of cutting.

Cutting	Factor	D.F.	S.S.	V	F	Required for significance .05	.01
1	Total	116	1,154.68				
	Between groups	4	203.86	50.97	6.004**	2.46	3.51
	Error	112	950.82	8.49			
2	Total	98	1,185.24				
	Between groups	4	36.99	9.25	.741	5.66	13.57
	Error	92	1,148.25	12.48			
3	Total	97	2,218.46				
	Between groups	4	165.09	41.47	1.879	2.47	3.54
	Error	93	2,052.57	22.07			

**Highly significant

Table 6. Correlation between dark green leaf color and carotene content in four inbred lines and 2 hybrid populations.

Cutting	55 selfed	21 selfed	21 x 55	55 x 21	5 selfed	58 selfed	Pooled
2	-.014	-.185	-.421	-.066	-.282	-.284	-.210*
3	.392	-.120	-.571	-.299	-.543	-.305	-.133

*Significant at 5-percent level.

tings. Five classes were set up, class No. 1 being composed of plants of very dark green foliage and the other four classes of plants of successively lighter green color.

Table 6 shows the correlation coefficients calculated for individual cuttings of each plant group. All were negative with the exception of that calculated for the third cutting of plant 55. All coefficients obtained were nonsignificant. However, these results indicated that there was a trend for plants of the lighter shades of green to possess less carotene than the dark green plants. Correlation between color and carotene content was calculated pooling data for all plants used in the experiment. A correlation coefficient of -.210, significant at the 5-percent level was calculated for the second cutting; that obtained for the third cutting was -.133, and was nonsignificant (Figs. 4 and 5). This indicated that either of these two coefficients could have been obtained as a matter of chance.

It was determined by analysis of the data collected in this experiment that all alfalfa plants do not vary consistently in their carotene content from one cutting to the next. Therefore, it was decided to reanalyze the data collected by Hackerott (1946) from 13 alfalfa clones and their polycross progeny to determine if similar variations were present in his studies.

Tables 7 and 8 give the results of these analyses. Correlation coefficients calculated for all possible comparisons of the cuttings of each group show that in only two cases was a significant correlation found: between the 6/11/45 and 7/13/45 cutting

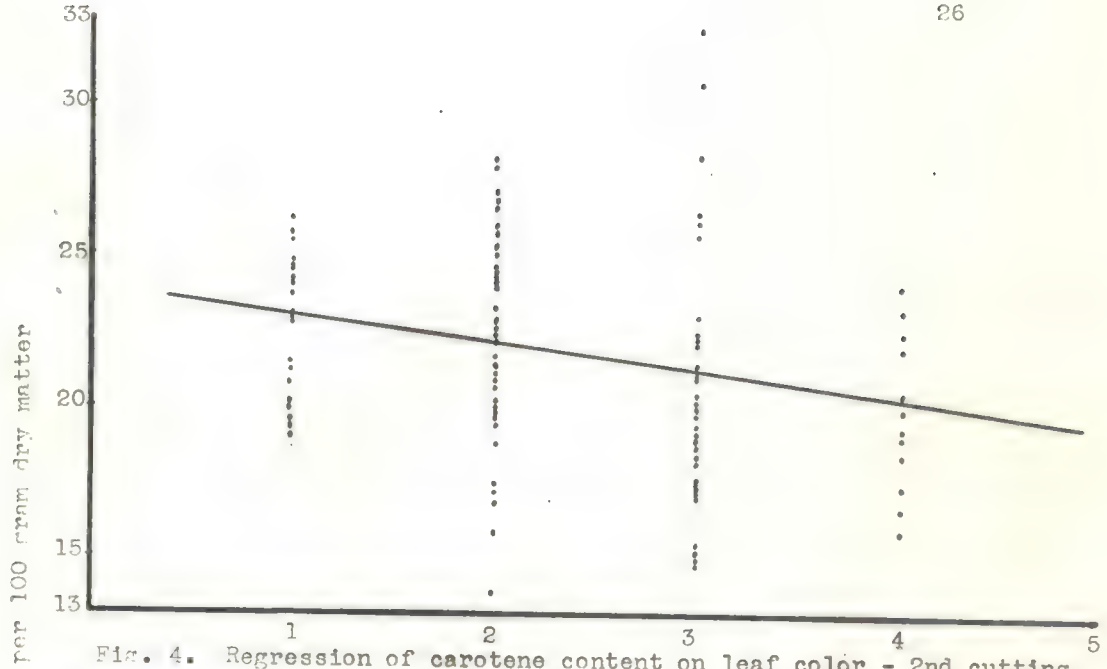

Fig. 4. Regression of carotene content on leaf color - 2nd cutting.

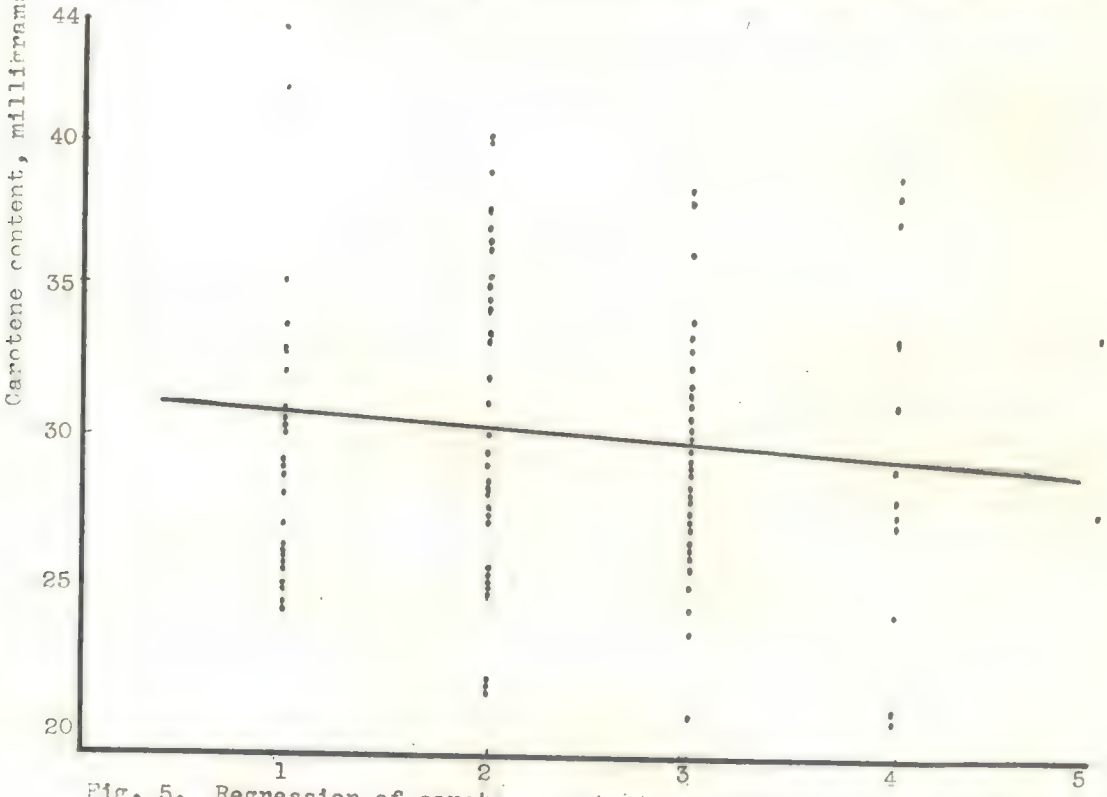

Fig. 5. Regression of carotene content on leaf color - 3rd cutting.

dates of the 13 parental clones, and the 2/28/45 and 6/5/45 cutting dates of their polycross progeny. It is significant to note that samples taken only two days apart, 2/26/45 and 2/28/45, did not show correlated carotene values. Both sets of samples were taken from rows of 50 seedlings of the 13 polycross progenies under study growing in the greenhouse bacterial wilt testing bed. Fresh material was used in carotene analysis of the samples taken on these two dates. The lack of correlation might be attributed largely to sampling error as it is extremely difficult to obtain a homogeneous sample from plant material which has not been dried and ground.

Even though significant correlation was not consistently found, enough correlation was present to be combined in the totals to show significant differences among the groups being tested. It seems evident that although carotene content of alfalfa is highly subject to variability produced by various agencies, genetic differences exist which cause significant differences to appear between groups of different parentage.

Carotene content of the parental plants used was determined by Hackerott (1946). Plants 53, 58, and 63 were lost after the first cutting. Correlation calculations were carried out to determine if the carotene content of the parental plant as obtained by Hackerott (1946) showed significant relationships with the first cutting values obtained in 1945 from the same plants, and with the first cutting means of the selfed progeny. No significant correlation was found. Nonsignificant associations were also

Table 7. Correlation between the carotene content on different cutting dates of 13 parental clones studied by Mackerott (1946).*

Cutting dates compared			Correlation coefficient
8/24/44	vs	9/19/44	.439
"	"	9/27/44	.207
"	"	6/11/45	.532
"	"	7/13/45	.406
"	"	9/18/45	-.170
9/19/44	"	9/27/44	.321
"	"	6/11/45	.131
"	"	7/13/45	.317
"	"	9/18/45	-.177
9/27/44	"	6/11/45	.541
"	"	7/13/45	.249
"	"	9/18/45	.073
6/11/45	"	7/13/45	.658**
"	"	9/18/45	.447
7/13/45	"	9/18/45	.424

*.553 required for significance at the 5-percent level and .684 at the 1-percent level.
**Significant at the 5-percent level.

Table 8. Correlation between the carotene content on different cutting dates of the polycross progeny of the 13 parental clones studied by Mackerott (1946).*

Cutting dates compared			Correlation coefficient
2/26/45	vs	2/28/45	.446
"	"	6/5/45	.320
"	"	8/20/45	.316
"	"	10/1/45	.093
2/28/45	"	6/5/45	.582**
"	"	8/20/45	.426
"	"	10/1/45	.171
6/5/45	"	8/20/45	.155
"	"	10/1/45	.097
8/20/45	"	10/1/45	-.455

*.553 required for significance at the 5-percent level and .684 at the 1-percent level.
**Significant at the 5-percent level.

found when carotene content of the parental plants for the first two years, 1946 and 1948, were compared with the first cutting means of their selfed progenies.

DISCUSSION

It was found by analysis of variance of carotene content of the four inbred lines from plants 5, 21, 55, and 58 that measurable differences in the amount of variation shown existed. The variation present in the selfed progenies of plants 5, 55, and 58 was found to be nonsignificant, even though wide differences were present in the various cuttings of each of these plant groups. Similar variation existed in the separate cuttings of the selfed progeny of plant 21; however, these differences were combined in the totals to form significant variation when the data were analyzed. This difference in significance of variation in the selfed progeny of plant 21 apparently must be due to causes other than those found in the other three groups, assuming that environmental influence on all four groups was the same. This could be explained by assuming heterozygosity of plant 21 for the factors present for carotene content expression and a relative degree of homozygosity for these genes in the other three populations. It was observed in Table 2 that the two plants of highest carotene content were members of the 21 selfed population. This could be due to segregation in plant 21. The variation present in the selfed progeny of plant 58, although nonsignificant, approached such at the 5-percent level. High individuals were produced in this population also. Thus, it

seems that plant 55 could possess a degree of heterozygosity somewhat less pronounced than that present in plant 21.

In reciprocal crosses, it is expected that variation within the two hybrid groups will be approximately the same if only genic differences are involved. The calculated F value for within group differences in the cross 55 x 21 was nonsignificant while that present in the hybrids whose female parent was plant 21 (21 x 55) was found to be highly significant. It is logical to assume that an additional cause of variation is present in the latter cross which does not exist in its reciprocal cross 55 x 21. The differences which were obtained in the selfed progeny of plant 21 and in the hybrid progeny of which 21 was the female parent, might be explained by assuming a cytoplasmic influence as well as genic differences combining to produce a variable population expression of carotene content.

The results of correlation studies made support these conclusions. It was reported that complete correlation in carotene content from one cutting to the next was found only in the selfed progeny of plant 21. Highly significant correlation was present between the first and second cuttings of the cross 21 x 55, with correlation among the first and third and second and third cuttings approaching significance at the 5-percent level (Table 4). The selfed progeny of plant 55 and hybrids which had this plant as their female parent showed significant correlation between the first and third and the second and third cuttings respectively, with other comparisons in both groups falling well below signifi-

ance at the 5-percent level. Correlation in the selfed progeny of plant 21 could be explained by its assumed heterozygous nature, but the differences present between the reciprocal crosses of plant 21 and plant 55 can apparently be interpreted only by introduction of some cause of variation transmissible only through the female parent.

Analysis of the data obtained indicated that a significant association did not exist in the plants used in this experiment between dark green leaf color and carotene content. Atwood and Orum (1940), in a review of the literature concerned with advancements in the genetics of alfalfa, reported that Schreck found the intensity of green color in alfalfa leaves to be controlled by a single major factor. Limited information suggest that the total amount of carotene in the alfalfa plant might be controlled by genic factors. The results of this work indicate that cytoplasmic influence might also be present in some cases. If intensity of green color in alfalfa foliage is controlled by one dominant factor, it seems unlikely that there would be an association of color and carotene content unless some of the factors for carotene content expression were linked with the color factor. The results obtained indicate that a nonsignificant trend existed among the plants used in this experiment, for those of darker green color to possess more carotene than the lighter green plants. Thus, dark green leaf color should not be used as a single criteria upon which to base selection of alfalfa plants for high carotene content.

Variability within the plant groups studied in this experiment increased with cuttings. If the view is taken that young rapidly growing plants are more susceptible to environmental fluctuations than are established plants, then their inherent potentialities for carotene production could be more easily masked than those of older plants. The influence of environment on carotene content of alfalfa is so great that differences are hidden even though the plants of the population under consideration might be genetically different in regard to the factors for carotene content expression. For this reason, in order that an accurate study of the carotene content of alfalfa may be made, only plants which have been established for a year or more should be used. It is suggested that these plants might be set out in clonal lines. This would enable the experimenter to take multiple samples each time the plants were cut. Only one sample per cutting was obtainable in this experiment. Statistical results were thus weakened, as different sampling dates were not considered to be replications.

Hsu and Tysdal (1946) found that about 77.1 percent of the carotene of the alfalfa plant was located in the leaves. They also reported that there was little variation between the carotene content of the stems of young and old alfalfa plants. Muir-crott (1946) decided that differences in carotene content between plants evidently were due to differences in concentration of carotene in the leaves of the plant concerned as well as to a changing leaf-stem ratio. From a plant breeding standpoint, samples ob-

tained from mixtures of leaves and stems probably give an accurate picture of the total carotene as it actually exists in the alfalfa plant. However, since the constant carotene content of the stem will effect the changing concentration within the leaves, leaf analysis should also be run in order to determine the extent of these differences in concentration.

The complexity of the problem of carotene content in alfalfa has been emphasized by the results of this experiment. The inheritance of carotene is probably controlled by interacting genetic and environmental factors, and the possibility exists that cytoplasmic influences may be involved. It is considered that segregates have been obtained in this experiment which will be useful in the continuation of the study of the inheritance of carotene content of alfalfa.

SUMMARY

Evidence has been obtained indicating that the parental plants used in this study varied in their genetic constitution of the factors controlling carotene content in alfalfa. In addition, it is believed that parental plant 21 might contain a cytoplasmic factor influencing carotene as different degrees of significance were obtained in which it was used as the male and female parent in reciprocal crosses.

The results of correlation studies made between dark green leaf color and carotene content indicate there was no significant relationship existing in the plants used in this experiment. The

use of this factor alone as a criteria of high carotene content in alfalfa is not recommended.

The inheritance of carotene content in alfalfa probably rests on complex interaction between environment and heritable factors within the plant. It is considered that segregates have been obtained which will be useful in a continuation of the study of the inheritance of carotene content of alfalfa.

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